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AD-A207 048

CONTRACT NO.: 86PP6813

TITLE: THE REGULATION OF A POST-TRANSLATIONAL PEPTIDE ACETYLTRANSFERASE: STRATEGIES FOR SELECTIVELY MODIFYING THE BIOLOGICAL ACTIVITY OF NEURAL AND ENDOCRINE PEPTIDES

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REPORT DATE: FEBRUARY 1, 1989

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21701-5012

89 3 27 110

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Uniformed Services University of the Health Sciences 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 4301 Jones Bridge Road Bethesda, MD 20814-4799	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER 86PP6813
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102A PROJECT NO. 3M1-61102BS12 TASK NO. AC WORK UNIT ACCESSION NO. 109

11. TITLE (Include Security Classification)
The regulation of a post-translational peptide acetyltransferase: Strategies for selectively modifying the biological activity of neural and endocrine peptides.

12. PERSONAL AUTHOR(S)
Millington, William R.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 88/2/1 TO 89/1/31	1989, February 1	31

16. SUPPLEMENTARY NOTATION

17. COSATI CODES

FIELD	GROUP	SUB-GROUP
06	01	
06	15	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Post-translational processing; Acetylation; beta-endorphin; pro-opiomelanocortin; pituitary; peptide; dopamine; RA 1

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
The broad objective of this research is to develop new strategies for pharmacologically modifying synaptic transmission by peptidergic neurons. It is based on the principal that post-translational processing defines the biological activity of neuropeptides and uses the β-endorphin processing pathway as a model for study. Two major objectives were completed during this project period. First, we examined the functional consequences of β-endorphin processing, demonstrating that C-terminal shortening of β-endorphin-1-31 to β-endorphin-1-27 augments its central hypotensive potency. This contrasts studies on analgesia where β-endorphin-1-27 is a weak agonist, but a highly potent antagonist, and indicates that peptide processing can produce entirely different changes in bioactivity depending upon the postsynaptic receptors which mediate the response. The second objective characterized β-endorphin processing in human brain, demonstrating that β-endorphin-1-31 is the predominant form; significant amounts of β-endorphin-1-27 and -1-26 and small amounts of N-acetylated β-endorphin peptides were also present. Thus, despite important differences in primary structure, β-endorphin is processed similarly in both human and rat brain, thereby confirming the rat as a valid model.
(over)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued)

for investigating the function and regulation of β-endorphin processing. These findings provide further support for the concept that chemical agents designed to alter the activity of β-endorphin processing enzymes will have important effects on the physiology of brain β-endorphin and may be of significant clinical utility.

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FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Feb. 1, 1989

PI Signature Date

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INTRODUCTION

The discovery that biological peptides act as synaptic neurotransmitters initially offered considerable promise for the development of a whole new generation of centrally active drugs. But the prospect of using peptides as templates for drugs has born little fruit, primarily because peptides do not permeate the blood-brain barrier or other biological membranes and because they are rapidly metabolized. These limitations prompted us to consider whether a closer evaluation of the pre-synaptic mechanisms which govern peptide biosynthesis and post-translational processing might lead to alternative approaches for designing therapeutic agents to modify synaptic transmission by peptidergic neurons. The broad objective of this research program is, therefore, to create the data base necessary to develop new strategies for pharmacologically modifying the function of peptidergic neurons and endocrine cells.

Most neuropeptides are initially synthesized as large, biologically inactive precursors which must undergo enzymatic processing to yield their biologically active peptide products. Studying the enzymatic processing of peptide precursors can be exceedingly complex, however; for example, pro-opiomelanocortin (POMC), which for many years has served as a prototype for studying peptide biosynthesis, is enzymatically processed to over twenty different peptides (O'Donohue and Dorsa, 1982; Millington and Chronwall, 1989). For this reason, we focused our research on the post-translational processing of β-endorphin, a product of POMC. β-endorphin is initially synthesized as a thirty-one amino acid peptide, which is further processed to both N-acetylated and des-acetyl β-endorphin-1-31, -1-27 and -1-26 (Fig. 1) (Zakarian and Smyth, 1979; Eipper and Mains, 1981; Millington et al., 1987). The sequential processing of β-endorphin is heterogeneous, which means that all of these molecular forms co-exist, in varying proportions, in the brain, pituitary and certain peripheral tissues (Zakarian and Smyth, 1982; Dennis et al., 1983; Emeson and Eipper, 1986).

The β-endorphin processing pathway clearly illustrates the principle that enzymatic processing determines biological activity. Evidence for this initially arose from studies of β-endorphin's analgetic activity. These experiments demonstrated that both N-acetylation and C-terminal proteolysis essentially eliminate the potent analgetic properties of β-endorphin-1-31 (Deakin, et al., 1980; Nicolas and Li, 1985). Moreover, C-terminal cleavage converts β-endorphin-1-31 to a potent opioid receptor antagonist, β-endorphin-1-27. β-endorphin-1-26, on the other hand, is devoid of either agonist or antagonist activity. Thus, relatively minor changes in structure produce profound changes in bioactivity.

We initiated studies of β-endorphin processing by asking the basic question, 'are post-translational processing enzymes regulated?' This is a fundamental question because closely regulated enzymes are the rate limiting steps in biosynthetic

pathways and commonly provide effective target sites for drug action. We began by using the intermediate pituitary to study the regulation of peptide acetyltransferase, the enzyme which N-acetylates β-endorphin. The intermediate lobe is an ideal model system. A homogeneous tissue, controlled primarily by inhibitory dopaminergic neurons, it processes β-endorphin in a pattern similar to that of brain (Millington and Chronwall, 1989). We found that peptide acetyltransferase is indeed regulated; chronic treatment with dopaminergic drugs produced coordinated changes in peptide acetyltransferase activity, POMC gene expression and β-endorphin secretion (Millington et al., 1986). We also found, however, that carboxypeptidase H, the enzyme thought to convert β-endorphin-1-27 to 1-26, is not similarly regulated. Moreover, the differential regulation of these two enzymes induced selective changes in the molecular forms of β-endorphin released from the intermediate lobe (Millington et al., 1987). These experiments clearly demonstrated that peptide processing enzymes are individually regulated and established the feasibility of using pharmacological agents acting at synaptic receptors to selectively control the processing, and hence the biological activity, of β-endorphin peptides.

These studies also revealed that the regulation of β-endorphin processing is but one component of a series of temporally related intra- and intercellular mechanisms used by the intermediate lobe to modify POMC biosynthesis. We combined several coordinated methodological approaches to investigate intermediate lobe function, including dot blot and in situ hybridization, light and electron microscopic morphometric analysis, ^{3}H-thymidine uptake measurement and radioimmunoassay (Chronwall et al., 1987 & 1988; Millington and Chronwall, 1989). This revealed that acute treatment with dopamine receptor antagonists accelerates POMC gene transcription, elevating POMC mRNA levels, and increasing both the synthesis and secretion of β-endorphin. Subchronic treatment continues to accelerate POMC biosynthesis and also converts biosynthetically quiescent 'light' cells into 'dark' cells, those actively engaged in synthesizing POMC, thus further expanding the biosynthetic capacity of the gland as a whole. Following chronic treatment, the intermediate lobe exhibits cellular hyperplasia, an increase in the number of cells in the gland. Finally, after extended periods of interrupted dopaminergic innervation, the rate of POMC synthesis within individual intermediate lobe cells returns to control levels and cellular hyperplasia alone maintains elevated β-endorphin output from the gland (Millington et al., 1988; Dybdal et al., 1988). Thus, the intermediate lobe utilizes several independent, yet coordinated, mechanisms to modulate its total output of peptide hormones. Interestingly, alterations in β-endorphin processing occur only during chronic conditions. The selective regulation of β-endorphin processing apparently provides a mechanism for encoding information about the temporal nature of environmental stimuli, specifically, certain stressful conditions (Berkenbosch et al., 1984), perceived by the central nervous system and transmitted to peripheral tissues, including the immune system, by the intermediate pituitary.

Upon completing investigations of the endocrine β-endorphin system, we initiated studies of β-endorphin processing in brain. Our first objective was to characterize the distribution and regulation of β-endorphin peptides. Briefly, we found that all the β-endorphin forms identified in the pituitary are present in brain, although their relative proportions vary among brain regions. This indicates that peptide acetyltransferase and other β-endorphin processing enzymes are expressed in brain and that their regulation is regionally specific. Furthermore, the enzymes apparently can be controlled in brain, as in the pituitary, by synaptic activation, based on the finding that dopaminergic drugs also modify brain β-endorphin processing; however, other pharmacologic treatments thought to produce changes in POMC biosynthesis, including chronic morphine or estradiol administration, were ineffective. Neuronal β-endorphin processing also changes physiologically, during both early maturation (Martensz, 1985) and the aging process (Wilkinson and Dorsa, 1986) although the specific modifications differ from those occuring in the pituitary (Alessi et al., 1983; Martensz, 1985). Together, these findings suggest that the regulation of neuronal β-endorphin processing differs from that of the pituitary (Berglund et al., 1989).

The observation that β-endorphin processing can be altered both physiologically and pharmacologically prompted us to study the functional consequences of these regulatory processes. The role of processing in mediating the analgetic activity of β-endorphin is well understood (Nicolas and Li, 1985); however β-endorphin neurons are not solely involved in analgesia, but mediate other physiological and behavioral responses as well (O'Donohue and Dorsa, 1982). To address this question, we initiated studies of the central cardiovascular effects of β-endorphin peptides. This, the second objective of our study of neuronal β-endorphin processing, will be described in detail in the Results and Discussion section. The third objective questions the ultimate clinical applicability of our basic studies using laboratory animals. While our findings suggest that pharmacologic agents targeted on β-endorphin processing enzymes may have potential clinical utility, this relies on the assumption that the β-endorphin processing pathway is the same in human brain as that of laboratory animals. Critical species differences in the primary structure of β-endorphin suggests that this may not be the case, however (Li, 1984). We therefore initiated studies of the β-endorphin processing pathway in human brain. Substantive progress has been made toward completing both objectives during this project period.

RESULTS AND DISCUSSION

Central Cardiovascular Effects of β-Endorphin Peptides

One of the most intriguing aspects of β-endorphin processing is that relatively minor post-translational changes in peptide structure produce profound changes in biological activity. As discussed previously, the potent analgetic properties of β-endor-

phin-1-31 are essentially abolished by both N-acetylation and C-terminal proteolysis. Moreover, in addition to substantially reducing agonist activity, C-terminal proteolysis produces an entirely different property; it converts β-endorphin-1-31 from an agonist to a highly potent opioid receptor antagonist, β-endorphin-1-27 (Nicolas and Li, 1985). Indeed, β-endorphin-1-27 is four to five times more potent than the classical opiate antagonist, naloxone. Receptor binding studies also showed that β-endorphin-1-27 displaces both etorphine and β-endorphin-1-31 binding, further evidence that the peptide functions as an antagonist at mu and/or delta opioid receptors (Akil et al, 1981; Nicolas and Li, 1985). These intriguing findings prompted us to raise a broader, rather fundamental question; does β-endorphin-1-27 block other physiologic and behavioral responses mediated by β-endorphin neurons or is the relationship between processing and biological activity functionally specific? To address this question we initiated studies of the role of β-endorphin processing in defining the central cardioregulatory properties of the peptide.

To study the central cardiovascular effects of β-endorphin, we administered β-endorphin-1-31 or -1-27 intracisternally (ic) to chloralose-anesthetized rats and recorded mean arterial pressure (MAP), pulse pressure and heart rate directly through an arterial cannula. Our initial experiments demonstrated, as others had shown previously, that β-endorphin-1-31, like morphine, lowers blood pressure (Petty and de Jong, 1982; Holaday, 1983). MAP was reduced by approximately 30 mm Hg 60 min after ic injection of 1.5 nmol β-endorphin-1-31 and returned toward baseline values by 120 min (Fig. 2). Unexpectedly, β-endorphin-1-27 also produced a potent hypotensive response; indeed, its potency was 5-10 fold greater than β-endorphin-1-31 (Hirsch et al., 1988). β-endorphin-1-27 (1.5 nmol) lowered MAP to a significantly greater extent than an equimolar β-endorphin-1-31 dose while 0.15 nmol β-endorphin-1-27 produced a response essen-tially equivalent to 1.5 nmol of the parent peptide (Fig. 2, Table 1). β-endorphin-1-27 also produced bradycardia but only at the higher dose tested (1.5 nmol); neither β-endorphin-1-31 nor the lower dose of β-endorphin-1-27 had any effect on heart rate (Fig. 3). Respiration was not affected by either peptide. Thus, C-terminal proteolysis potentiates the hemodynamic actions of β-endorphin while greatly reducing its analgetic potency, converting it to an opioid receptor antagonist. This clearly demonstrates that post-translational processing subserves quite different roles in defining the analgetic and cardioregulatory responses produced by β-endorphin peptides.

Next we examined the hemodynamic potency of the other four β-endorphin forms, β-endorphin-1-26 and N-acetyl-β-endorphin-1-31, -1-27 and -1-26. This revealed that all four peptides were completely devoid of either hypotensive or bradycardic activity (Figs. 4 and 5, Table 1). Thus, while the conversion of β-endorphin-1-31 to -1-27 enhances hemodynamic potency, both N-acetylation and further C-terminal proteolysis to β-endorphin-1-26 inactivate the peptide. These processing steps therefore produce the same effect on both analgesia and hypotension; they eliminate bioactivity.

Finally, we tested whether the hemodynamic action of β-endorphin peptides could be inhibited with the opioid receptor antagonist, naloxone. We found that indeed it was; naloxone pretreatment (300 nmol, ic) completely blocked the effects of both β-endorphin-1-31 and β-endorphin-1-27 on MAP and heart rate but produced no hemodynamic effects when given alone (Table 1). This indicates that the cardioregulatory effects of both peptides are mediated by opioid receptors.

Clearly, C-terminal proteolysis is a critical determinant of the pharmacological activity spectrum of β-endorphin peptides. However, to demonstrate the physiologic relevance of these pharmacologic data, it is essential to determine whether these pharmacologically active β-endorphin forms are actually present in brain. We therefore characterized the molecular forms of β-endorphin present in the nucleus of the solitary tract (nTS), an area importantly involved in the central regulation of cardiovascular function (Holaday, 1983). The nTS β-endorphin processing pathway is of particular interest because a relatively dense population of β-endorphin containing neuronal perikarya are localized within the nucleus; indeed, it is the only brain region outside of the medial basal hypothalamus where endorphinergic cell bodies are found (Joseph et al., 1983; Pilcher and Joseph, 1986; Palkovits et al., 1987). Furthermore, microinjection of β-endorphin-1-31 directly into the nTS lowers blood pressure (Petty and de Jong, 1982), suggesting that exogenously administered β-endorphin peptides act through receptors normally activated by β-endorphin released from nTS neurons. To examine the β-endorphin processing pathway in the nTS, we microdissected the nucleus using the Palkovits technique (Palkovits et al., 1987), pooling tissue from approximately one hundred animals, and separated β-endorphin peptides by cation exchange chromatography (Millington et al., 1987). This revealed that β-endorphin-1-31 is the quantitatively major form in the nTS, constituting 52% of total immunoreactivity. Relatively large amounts of β-endorphin-1-27 (21%) were also present but N-acetyl-β-endorphin-1-31 (5%), -1-27 (13%) and -1-26 (8%) were relatively minor products. These results show that the forms of β-endorphin wnich produce hypotension when injected in pharmacologic doses are, indeed, synthesized in the nTS.

The mechanism responsible for the differential analgetic and cardioregulatory effects of β-endorphin-1-27 remains to be established but the data are consistent with evidence that β-endorphin peptides exert their diverse effects by activating several different opioid receptor subtypes. The analgetic activity of β-endorphin-1-31 is thought to be mediated by mu and/or delta opioid receptors (Pasternak, 1987) and there is strong evidence that β-endorphin-1-27 also acts by blocking these same receptor subtypes (Nicolas and Li, 1985). Additional evidence further suggests, however, that certain β-endorphin induced responses are mediated by a third opioid receptor subtype, the epsilon receptor. Data supporting this concept is derived, in part, from receptor binding experiments using brain tissue (Goodman et al., 1983; Houghten et al., 1984), but also from physiological studies of peripheral

tissues, including the rat vas deferens and guinea pig ileum, that have served as model systems for opiate research for many years (Schulz et al., 1981; Huidobro-Toro et al., 1982; McKnight et al., 1983). These studies showed that β-endorphin-1-27 acts as an agonist at epsilon receptors; indeed, it's potency is, in some studies, even greater than that of β-endorphin-1-31. Furthermore, epsilon receptors are also blocked by naloxone. These properties are consistent with our studies of the hemodynamic effects produced by β-endorphin-1-27, suggesting that the peptide may produce hypotension by activating receptors similar to the putative epsilon opioid receptor. The differential effects of β-endorphin peptides on analgesia and cardiovascular regulation thus support the hypothesis that a single processing step, C-terminal proteolysis, produces entirely different changes in biological activity depending upon the postsynaptic receptors which mediate the response.

The Post-Translational Processing of β-Endorphin in Human Brain

The presence of β-endorphin immunoreactivity in human brain was first demonstrated soon after the initial discovery of the peptide (Gramsch et al., 1980; Emson, et al., 1984). Its distribution is similar to that of the rat and other mammals and, like the rat, most of the immunoreactive β-endorphin in human brain is localized within neuronal processes arising from cell bodies in the medial basal hypothalamus (Bugnon, et al., 1979). However, exactly how β-endorphin is processed in the human, whether to opiate active or inactive forms, is completely unknown. This information is essential for predicting whether the physiological effects and regulatory mechanisms we observed in the rat are relevant to the human. Moreover, our long term objective of developing clinically useful pharmacologic strategies for modifying brain β-endorphin processing demands that the animal species we study accurately models the human β-endorphin processing pathway. There is reason to believe that this may not be the case, however, because the primary structure of human β-endorphin differs in certain critical respects from the rat and virtually all other mammalian species (Li, 1984).

Before initiating these experiments we addressed a problem common to studies of human autopsy material; the effect of the postmortem interval, that is, the duration of time between death and freezing the tissue. To test whether the β-endorphin processing pattern is artifactually changed during the postmortem interval, we conducted experiments with rats which mimicked the treatment conditions for human autopsy material. We found that storing rat hypothalami, either at room temperature for 8 hours or at 4°C for 2, 6, 12 or 24 hours, had no effect whatsoever on the molecular forms of β-endorphin (Smith and Millington, 1989). Significant decrements did occur, however, in total β-endorphin immunoreactivity following storage at 4°C for six hours or longer. These results indicate that while some β-endorphin is lost during the postmortem interval, the overall processing pattern still reflects that present immediately after death.

Having established the feasibility of using human tissue, we proceeded to examine the molecular forms of β-endorphin in human hypothalamus. The samples were provided to us by the Brain Tissue Resource Center of McLean Hospital, an affiliate of Harvard Medical School. The subject population consisted of both males and females ranging in age from 23 to 89 years with no history of psychiatric or neurologic disease. β-endorphin peptides were isolated by cation exchange high performance liquid chromatography (HPLC) and β-endorphin immunoreactivity was measured in the chromatography fractions by radioimmunoassay. This revealed that β-endorphin-1-31 was the principal form, constituting 56.2 $\pm$ 5.3 percent of total immunoreactivity (Fig. 6, Table 2). β-endorphin-1-27 (16.6 $\pm$ 2.0%) and β-endorphin-1-26 (15.0 $\pm$ 2.1%) were also present but acetylated forms were quantitatively minor, each comprising approximately 5% of total β-endorphin. As one might predict, β-endorphin processing varied among individual samples, some containing relatively high levels of acetylated β-endorphin peptides, but these differences did not appear to be correlated with age, sex, cause of death or any other identifiable parameter (Fig. 6). We also identified the β-endorphin forms in subregions of the hypothalamus, including the preoptic and suprachiasmatic nuclei, and found the processing pattern in these areas to be similar to the whole hypothalamus, suggesting that regional differences do not occur within the hypothalamus (Fig. 7). Efforts to isolate β-endorphin peptides from the amygdala, nucleus accumbens, median eminence and cerebrospinal fluid were not successful owing to the very low levels of the peptide in these tissues. Preliminary experiments revealed no differences in hypothalamic β-endorphin processing between control and schizophrenic subjects.

These studies are the first to identify the β-endorphin processing pathway in human brain. The results show that, despite important differences in primary sequence, β-endorphin is processed similarly in both human and rat hypothalamus (Zakarian and Smyth, 1979; Emeson and Eipper, 1986; Berglund et al., 1989), thereby confirming that the rat is an appropriate model for studying the regulation of brain β-endorphin processing. They also provide evidence that the same, or similar enzymes process β-endorphin in both species. As in the rat, C-terminal proteolysis is the primary processing pathway in human hypothalamus; however, the predominance of N-acetylated β-endorphin peptides in rat brain stem, hippocampus, colliculae (Zakarian and Smyth, 1979), nucleus accumbens (Dennis, et al., 1983) and caudal medulla (Dores et al., 1986) raises the possibility that N-acetylation is the primary pathway in the corresponding human brain regions as well. These findings provide further support for the concept that chemical agents designed to alter the activity of β-endorphin processing enzymes will have important effects on the physiology of brain β-endorphin and may be of significant clinical utility.

<u>The C-Terminal Cleavage of β-Endorphin-1-27</u>

Preliminary experiments were initiated during this project

period to identify the enzyme which converts β-endorphin-1-27 to β-endorphin-1-26. Several findings directed our research toward this problem. First, C-terminal proteolysis of β-endorphin-1-31 to β-endorphin-1-27 and -1-26 is the predominant post-translational pathway in both rat and human brain. Secondly, the formation of β-endorphin-1-26 eliminates both the analgetic and hypotensive actions of β-endorphin-1-27 which, again, emphasizes the importance of this processing step in brain. Finally, studies of the regulation of β-endorphin processing enzymes indicate that, unlike peptide acetyltransferase, the enzyme which converts β-endorphin-1-27 to -1-26 is not regulated (Millington et al., 1987). This was an important finding which led to the hypothesis that post-translational processing enzymes are controlled through two different regulatory mechanisms (discussed in Millington and Chronwall, 1989); however, confirmation of this hypothesis requires identification of the β-endorphin-1-27 carboxypeptidase enzyme.

Earlier investigations raised the speculation that carboxypeptidase H, the carboxypeptidase B-like enzyme which removes C-terminal lysine and arginine residues following the endoproteolytic cleavage of peptide prohormones, (Fricker and Snyder, 1982; Hook and Loh, 1984) also cleaves the C-terminal histidine from β-endorphin-1-27, forming β-endorphin-1-26 (Eipper and Mains, 1981). At first, this seemed unlikely because there was little evidence to support the contention that carboxypeptidase B-like enzymes do, in fact, cleave C-terminal histidine residues. We therefore initiated our studies by conducting a rather simple, albeit indirect, experiment; we incubated N-acetyl-β-endorphin-1-27 with carboxypeptidase B, separating N-acetyl-β-endorphin-1-27 from -1-26 using cation exchange HPLC. Carboxypeptidase B exhibits a substrate specificity similar to that of carboxypeptidase H (Fricker, 1985) but has the added virtue of being readily available in purified form from commercial sources. This experiment revealed that carboxypeptidase B does, in fact, convert β-endorphin-1-27 to β-endorphin-1-26 although its apparent affinity for C-terminal histidine appeared to be several orders of magnitude lower than its affinity for arginine residues (Fig. 8). These experiments, while speculative, support the hypothesis that carboxypeptidase H may catalyze this processing step and encouraged us to proceed with our investigation.

The objective of these studies was to identify β-endorphin-1-27 carboxypeptidase activity in secretory vesicle fractions isolated from bovine intermediate lobe, then to characterize the enzyme activity to determine whether its properties were consistent with the known characteristics of carboxypeptidase H (i.e., substrate specificity, inhibitor profile, activation by Co^{+2}, molecular size, etc.) (Fricker, 1985) or if a different enzyme, previously unidentified, was responsible for this processing step. Thus far, we have completed the initial steps toward this objective. We first established a radiometric assay for carboxypeptidase H activity (Stack et al., 1984) with which to firmly establish that our secretory vesicle preparation contained sufficient amounts of active enzyme. We then characterized the molecular forms of β-endorphin in bovine intermediate lobe to ensure that β-endorphin-

1-26 is actually formed in this species. This showed that, while important species differences do occur, N-acetyl-β-endorphin-1-26 is synthesized in abundance, firm evidence that the enzyme is present. Thirdly, we conducted pilot experiments to isolated secretory vesicles from bovine intermediate lobe, scaling up methods previously established for separating secretory vesicles from rat pituitary (Millington et al., 1986). We then used this preparation to develop an assay for β-endorphin-1-27 carboxypeptidase activity. However, our initial attempts to identify the enzyme activity in this pilot study have met with variable success. Several potential causes have been identified: too little starting material; the low yield of our subcellular fractionation technique; the presence of large amounts of endogenous β-endorphin; the low sensitivity of the assay method. The solutions will require further scale-up and modification of the subcellular fractionation technique, removal of endogenous β-endorphin and the synthesis of [^{3}H]-β-endorphin-1-27 as an assay substrate (this is now being prepared through collaborative and commercial arrangements).

SUMMARY AND CONCLUSIONS

Central Cardiovascular Effects of β-endorphin Peptides: Studies completed during this project period demonstrated that post-translational processing defines the central cardioregulatory actions of β-endorphin. As shown previously (Petty and de Jong, 1982), β-endorphin-1-31, like morphine, is a potent hypotensive agent when administered centrally. β-endorphin-1-27, also lowers blood pressure; indeed its potency is 5-10 fold greater than β-endorphin-1-31 (Hirsch et al, 1988). This relationship is in marked contrast to analgesia where C-terminal shortening abolishes agonist activity, converting β-endorphin-1-31 from a highly potent agonist to an opioid receptor antagonist, β-endorphin-1-27 (Nicolas and Li, 1985). However, both further C-terminal proteolysis of β-endorphin-1-27 to β-endorphin-1-26 and N-acetylation have the same effect on analgesia and hypotension; they abolish bioactivity. These findings indicate that post-translational processing can produce either the same, or entirely different changes in peptide bioactivity depending upon the postsynaptic receptors which mediate the response. Thus, both pre- and post-synaptic mechanisms control the physiologic effects produced by β-endorphin neurons: pre-synaptic mechanisms regulate β-endorphin processing enzymes, producing different bioactive β-endorphin forms; post-synaptic mechanisms regulate the expression of opioid receptor subtypes, producing different responses to the same β-endorphin form.

The post-translational processing of β-endorphin in human brain: The presence of β-endorphin immunoreactivity in human brain was demonstrated over a decade ago but exactly how the peptide is processed, whether to opioid active or inactive forms, is completely unknown. This information is essential to establish whether the rat is a valid animal model for studying the function and regulation β-endorphin processing. We first established that artifactual changes in processing do not occur during the post-

morten interval by conducting experiments with rats which mimicked the treatment conditions for human autopsy material. We then isolated β-endorphin peptides from human hypothalamus using cation exchange HPLC. Opioid active β-endorphin-1-31 was the quantitatively major form although significant amounts of β-endorphin-1-27 and -1-26 are also present (Smith and Millington, 1989). N-acetyl β-endorphin peptides were quantitatively minor, together constituting approximately 15% of total immunoreactivity. A similar processing pattern was found in the preoptic and suprachiasmatic nuclei. These results demonstrate that, despite important differences in primary structure, β-endorphin is processed similarly in both human and rat hypothalamus, thereby confirming that the rat is an appropriate model for investigating the function and regulation of β-endorphin processing.

PERSONNEL

This project provides full salary support for the PI and a senior investigator, Dr. Michael D. Hirsch. In addition to the research described in this report, both the PI and Dr. Hirsch committed a portion of their efforts toward writing new grant proposals during this project period (Dr. Hirsch's position terminates with the cessation of funding for this project). Several manuscripts were also submitted, including a substantive review covering research supported by the project (Publications section, page 18). The PI also committed a limited amount of time to University teaching (6 contact hours) and committee work (approximately 2 hours per month).

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February 1, 1988 - January 31, 1989

Manuscripts:

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Millington, W.R. and Smith, D.L. The post-translational processing of β-endorphin in human brain. To be submitted to J. Neurochem.

Hirsch, M.D., Mueller, G.P. and Millington, W.R. β-Endorphin-1-27 is a potent endogenous hypotensive agent. To be submitted to J. Pharmacol. Exp. Ther.

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Smith, D.L. and Millington, W.R. The post-translational processing of β-endorphin in human hypothalamus. Winter Neuropeptides Conference, 1989.

Table 1. The hemodynamic effects of β-endorphin-1-31 and β-endorphin-1-27 are attenuated by N-acetylation, C-terminal proteolysis and by co-administration of naloxone.

Peptide	Dose (nmol)	MAP (mm Hg)	Heart Rate (Beats/min.)
β-End-1-31	1.5	-29.7 ± 3.9*	+17.8 ± 21.4
+ Naloxone		+4.7 ± 4.7	+4.0 ± 25.0
β-End-1-27	1.5	-47.3 ± 5.3**	-78.6 ± 17.2*
β-End-1-27	0.15	-25.9 ± 4.7*	-12.6 ± 12.8
+ Naloxone		-3.0 ± 2.0	+41.2 ± 15.3
β-End-1-26	1.5	-2.3 ± 1.3	+4.4 ± 8.0
Ac-β-End-1-31	1.5	+0.3 ± 5.0	+9.8 ± 11.4
Ac-β-End-1-27	1.5	+0.1 ± 3.2	+31.8 ± 10.8
Ac-β-End-1-26	1.5	+0.4 ± 1.6	+4.4 ± 3.3

β-endorphin peptides were injected intracisternally at the indicated dose and mean arterial pressure (MAP) and heart rate were recorded at 5 min intervals for two hours. Naloxone HCl (300 nmol) was also injected intracisternally, 30 min prior to peptide administration. The data are presented as the difference in MAP and heart rate between baseline, pretreatment values and those recorded 60 min following peptide administration. Control values for MAP and heart rate were 94.7 ± 1.9 mm Hg and 366 ± 15 beats per min, respectively. Naloxone alone had no effect on MAP (+3.2 ± 1.8 mm Hg) or heart rate (-6.0 ± 7.0 beats/min). The data represent the mean ± SE of 5 animals in each group. End = endorphin; Ac = acetyl. *, P<0.05; **, P<0.01 differs from control.

Table 2. The post-translational processing of β-endorphin in human hypothalamus.

Case Number	β-Endorphin Peptide (percent total immunoreactivity)					
	Ac-β-1-26	Ac-β-1-27	β-1-26	β-1-27	Ac-β-1-31	β-1-31
1	1.7	1.5	13.7	18.5	2.4	62.2
2	5.7	7.0	35.5	32.9	0	11.0
3	10.0	12.0	14.6	12.0	15.0	36.5
4	5.4	6.1	20.1	9.5	10.9	48.1
5	7.7	0	14.7	13.5	2.0	62.1
6	9.2	8.4	11.1	14.9	6.0	50.5
7	0	2.9	6.8	10.3	2.3	77.6
8	0.4	1.6	10.6	14.9	0.4	69.1
9	1.0	3.5	8.1	11.8	0	75.7
10	2.5	0	12.3	16.2	1.8	67.2
11	1.8	6.3	15.7	25.2	5.6	58.4
12	2.7	3.4	17.1	19.8	1.0	56.0
	4.0 ± 1.0	4.4 ± 1.1	15.0 ± 2.1	16.6 ± 2.0	4.0 ± 1.4	56.2 ± 5.3

Human hypothalami were homogenized in 1 N acetic acid, concentrated by Sep-Pak extraction and β-endorphin peptides were separated by cation exchange high performance liquid chromatography (HPLC) and analyzed by radioimmunoassay. The data are presented as the per cent of total immunoreactivity eluted from the cation exchange column for each individual sample. Ac = N-acetyl; β = β-endorphin.

FIGURE LEGENDS

Figure 1. The post-translational processing of β-endorphin in the intermediate pituitary of the rat.

Figure 2. C-terminal proteolysis of β-endorphin-1-31 enhances hemodynamic potency. Male Sprague-Dawley rats (250-300 g; n = 5) were anesthetized with chloralose and mean arterial pressure (MAP) was recorded at five minute intervals using a Gould model 30-V8202-10 physiograph equipped with a pressure transducer attached to a tail artery cannula. β-endorphin peptides were injected intracisternally (ic) dissolved in 5 μl artificial cerebrospinal fluid.

Figure 3. C-terminal proteolysis of β-endorphin-1-31 enhances bradycardic potency. Heart rate was recorded from groups of five rats as described in figure 2.

Figure 4. C-terminal proteolysis of β-endorphin-1-27 eliminates hemodynamic activity.

Figure 5. N-acetylation eliminates depressor activities of β-endorphin peptides. MAP was recorded at five minute intervals for ninety minutes as described in figure 2. The data represent the mean ± SEM (n = 5) recorded sixty minutes after peptide administration.

Figure 6. The post-translational processing of β-endorphin in human hypothalamus. Human hypothalami were homogenized in 1 N acetic acid, β-endorphin peptides were extracted using a Sep-Pak C-18 cartridge (Waters Corp.) and separated by cation exchange high performance liquid chromatography (HPLC) (Millington et al., 1987). The figure depicts HPLC chromatograms from six hypothalami.

Figure 7. The post-translational processing of β-endorphin in human suprachiasmatic and preoptic nuclei. β-endorphin peptides were separated by cation exchange HPLC as described in figure 6. The figure depicts a representative chromatogram from the suprachiasmatic nucleus (upper panel) and the preoptic nucleus (lower panel).

Figure 8. Carboxypeptidase B proteolysis of the C-terminal histidine residue of N-acetyl-β-endorphin-1-27. Carboxypeptidase B was incubated with N-acetyl-β-endorphin-1-27 (100 pmol) for 20 min in 120 μl 50 mM phosphate buffer (pH 7.6) containing 100 mM NaCl. N-acetyl-β-endorphin-1-27 and -1-26 were separated by cation exchange HPLC. Panel A depicts a control incubation in the absence of carboxypeptidase B. Panels B-D show the results of incubations with 0.034, 0.34 and 3.4 Units of carboxypeptidase B (1 U = 1 μmol hippuryl-L-arginine/min).

FIGURE 1

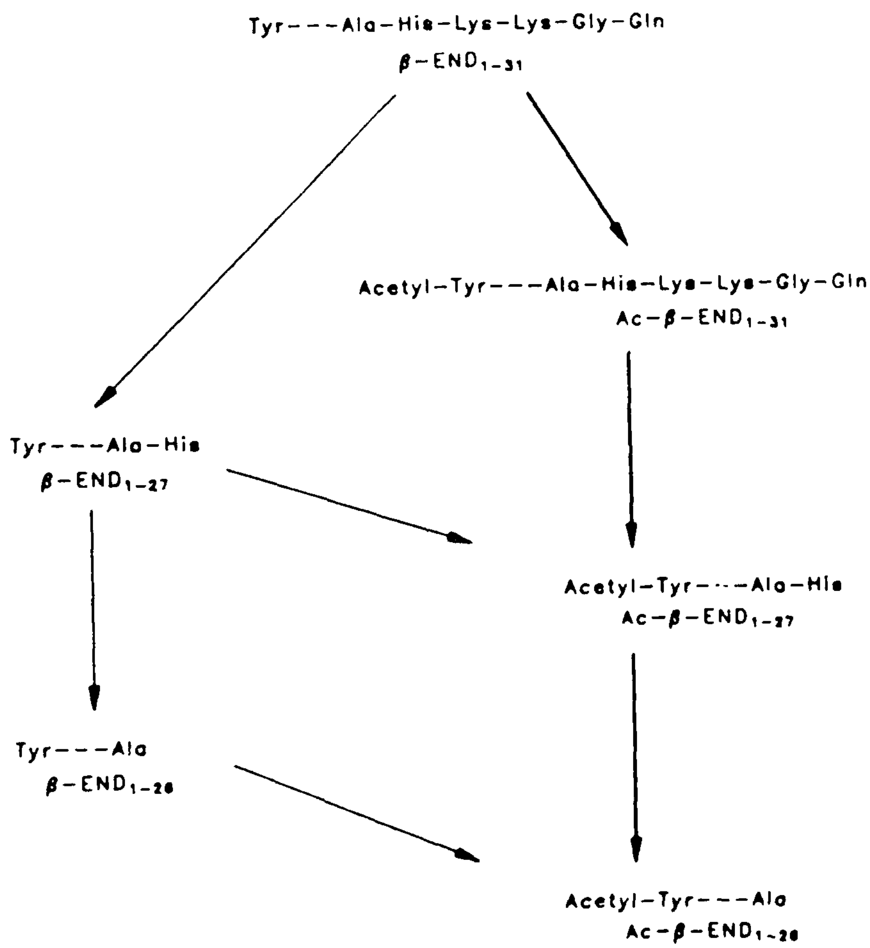

Tyr---Ala-His-Lys-Lys-Gly-Gln
β-END1-31
Acetyl-Tyr---Ala-His-Lys-Lys-Gly-Gln
Ac-β-END1-31
Tyr---Ala-His
β-END1-27
Acetyl-Tyr---Ala-His
Ac-β-END1-27
Tyr---Ala
β-END1-26
Acetyl-Tyr---Ala
Ac-β-END1-26

FIGURE 2

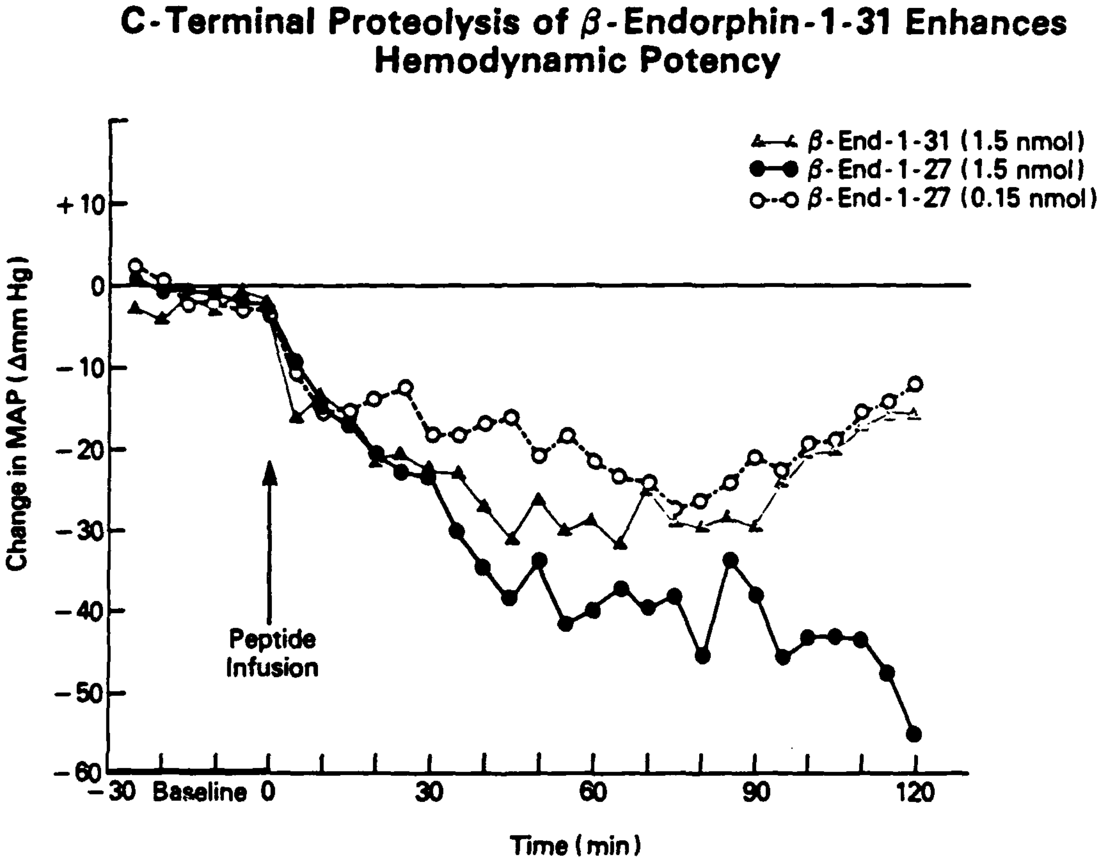

C-Terminal Proteolysis of β-Endorphin-1-31 Enhances Hemodynamic Potency
β-End-1-31 (1.5 nmol)
β-End-1-27 (1.5 nmol)
β-End-1-27 (0.15 nmol)
Change in MAP (Δmm Hg)
+10
0
-10
-20
-30
-40
-50
-60
Peptide Infusion
-30 Baseline 0
30
60
90
120
Time (min)

FIGURE 3

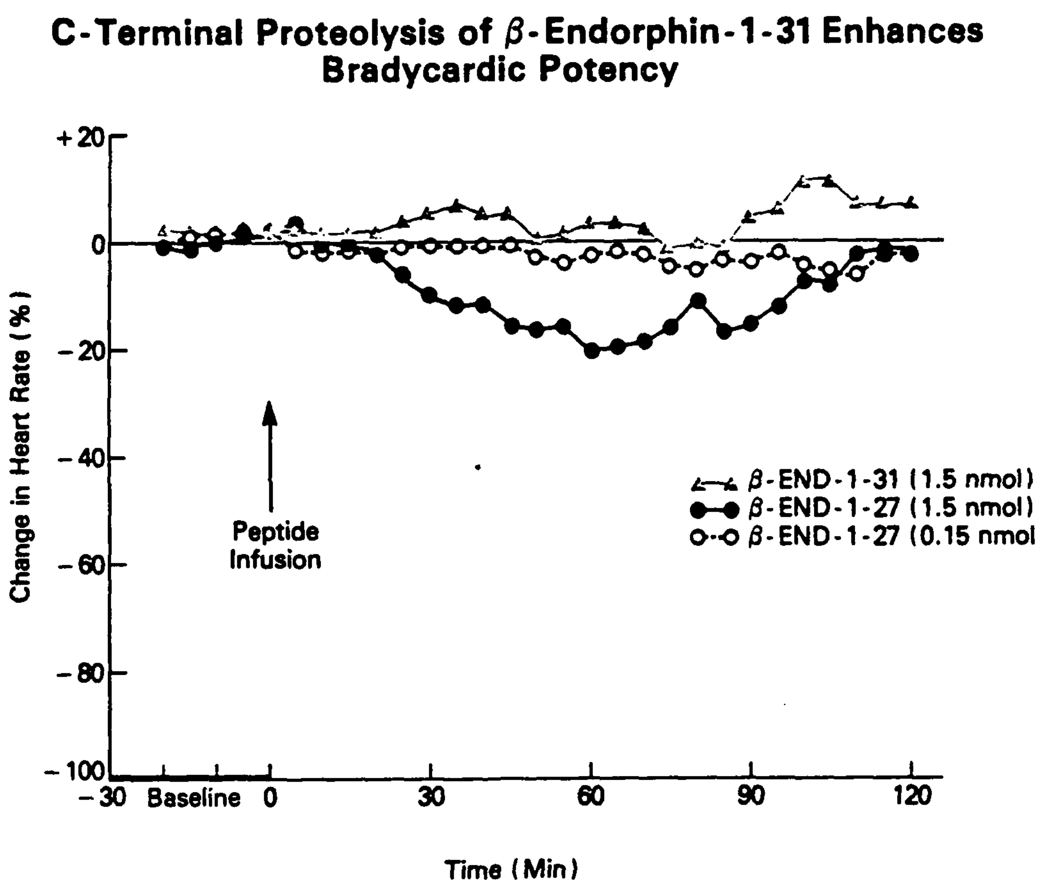

C-Terminal Proteolysis of β-Endorphin-1-31 Enhances Bradycardic Potency
+20
0
-20
-40
-60
-80
-100
Change in Heart Rate (%)
Peptide Infusion
β-END-1-31 (1.5 nmol)
β-END-1-27 (1.5 nmol)
β-END-1-27 (0.15 nmol)
-30
Baseline
0
30
60
90
120
Time (Min)

FIGURE 4

C-Terminal Proteolysis of β-Endorphin-1-27 Eliminates Hemodynamic Activity

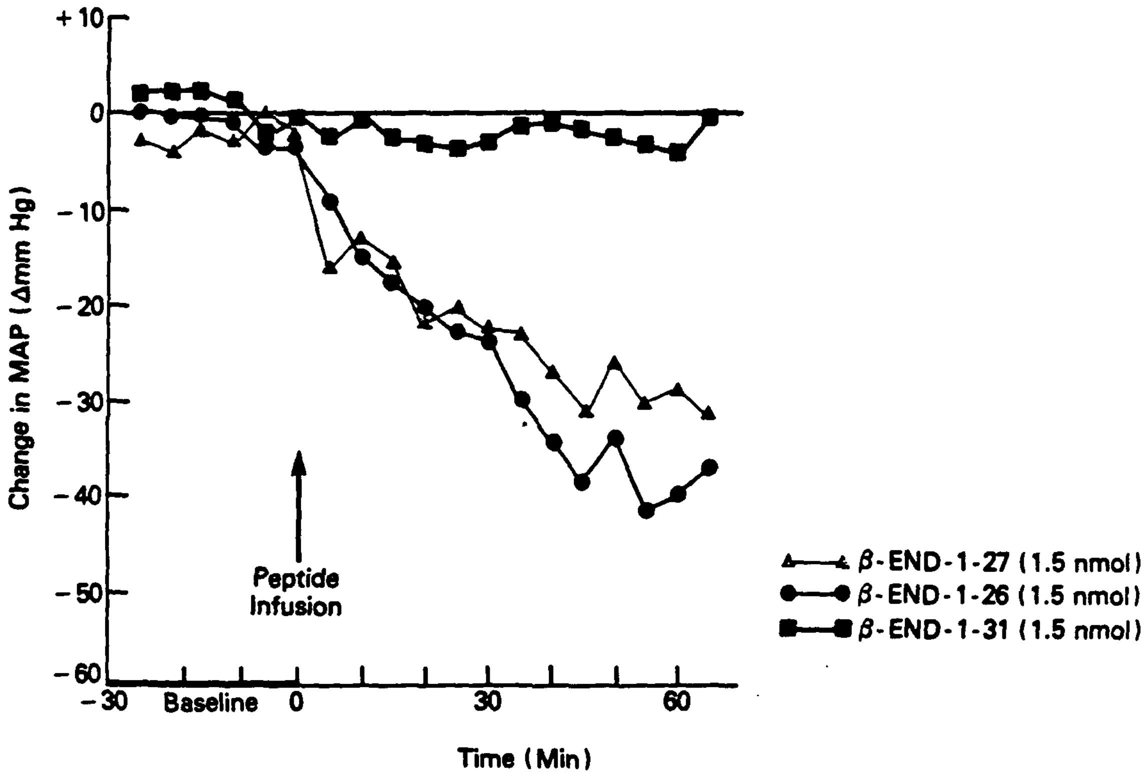


FIGURE 5

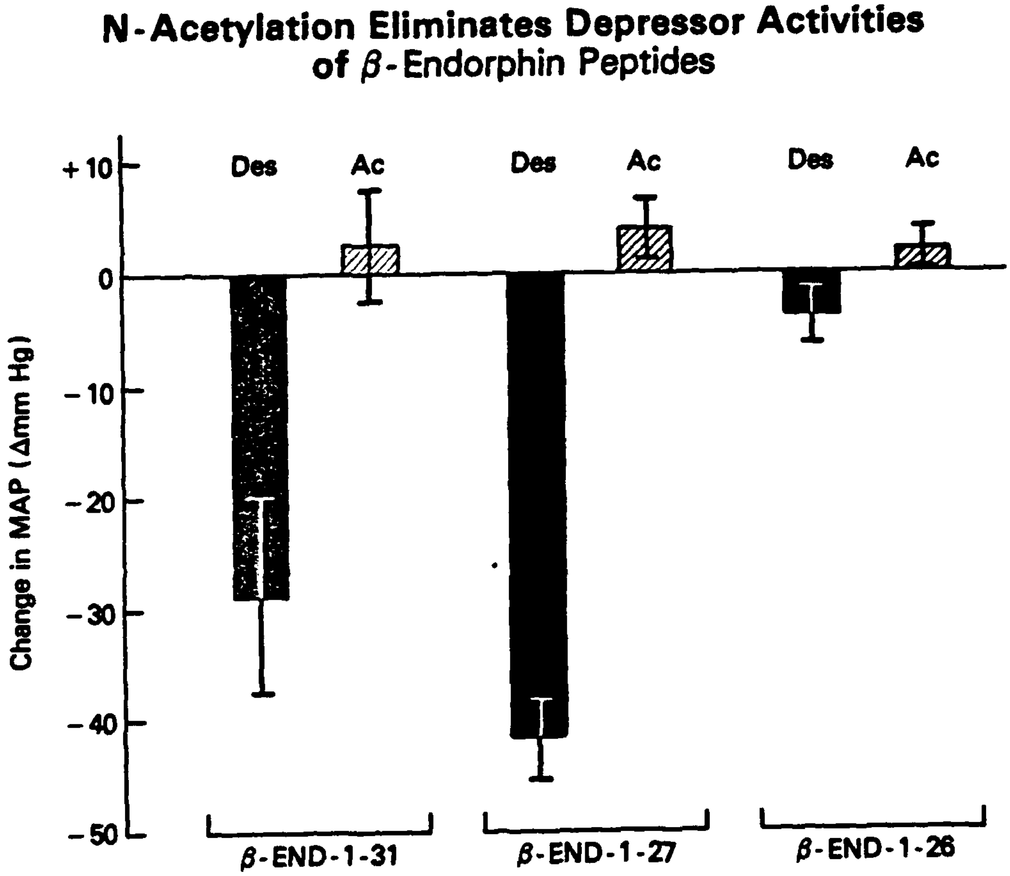

N-Acetylation Eliminates Depressor Activities of β-Endorphin Peptides
Change in MAP (Δmm Hg)
+10
0
-10
-20
-30
-40
-50
Des
Ac
Des
Ac
Des
Ac
β-END-1-31
β-END-1-27
β-END-1-26

FIGURE 6

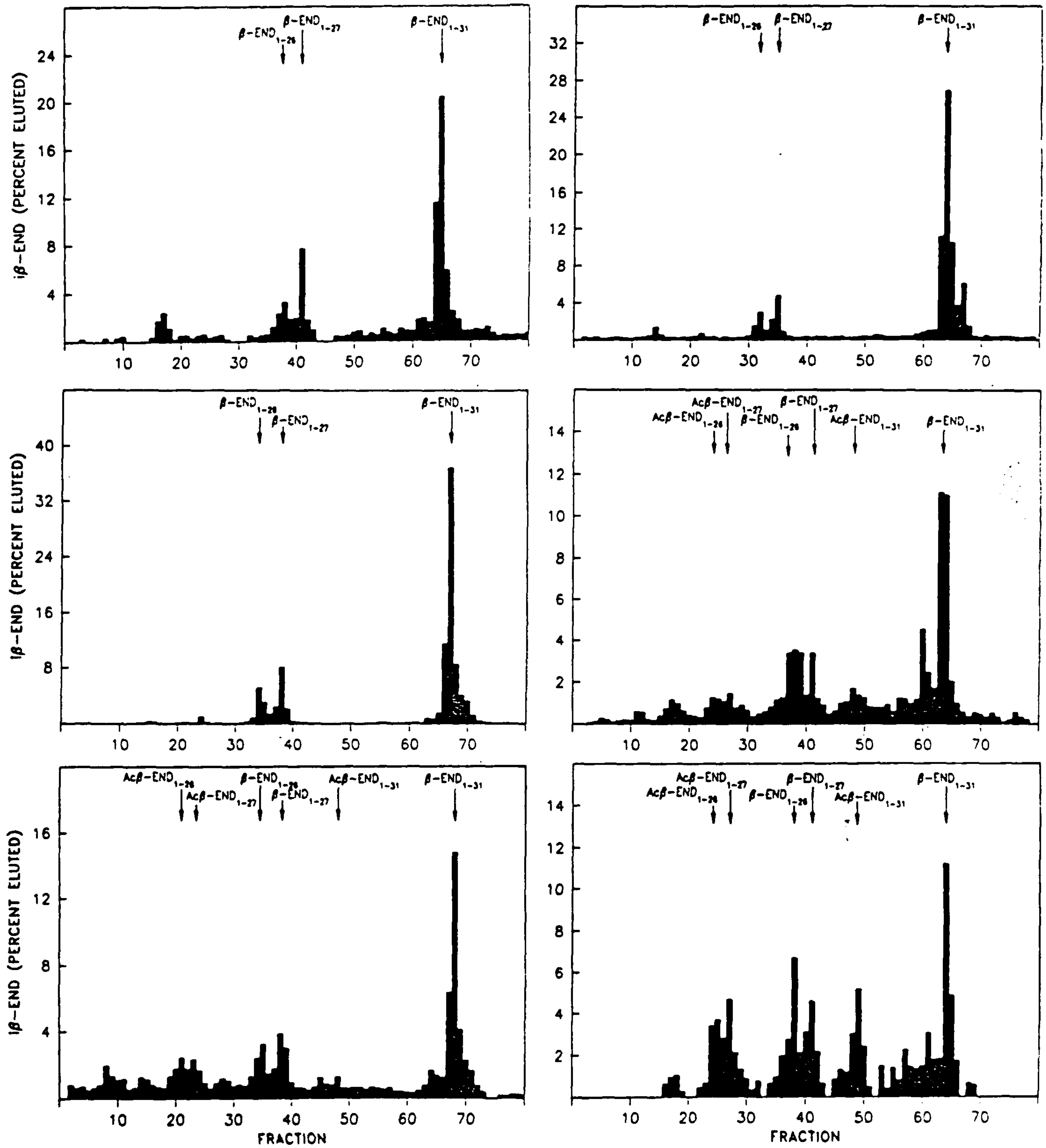

iβ-END (PERCENT ELUTED)
β-END1-26
β-END1-27
β-END1-31
AcβEND1-26
Acβ-END1-27
Acβ-END1-31
FRACTION

FIGURE 7

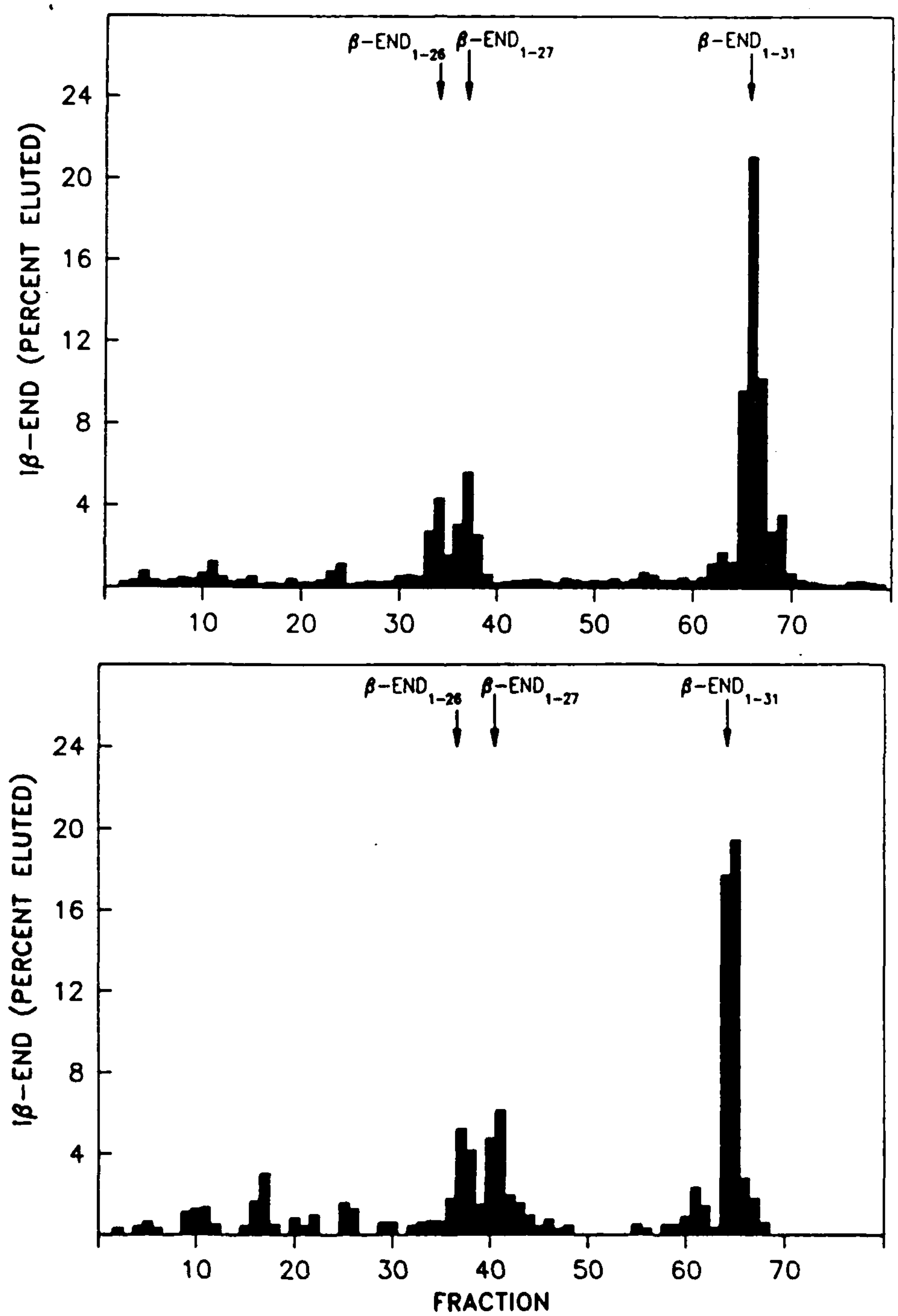
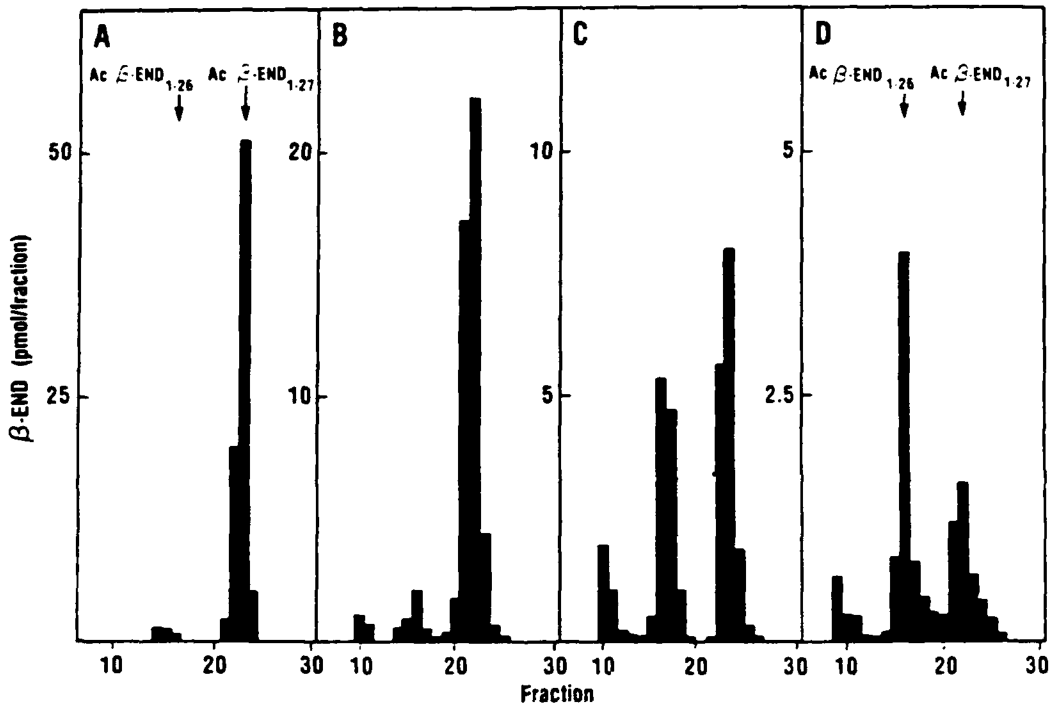

β-END1-26
β-END1-27
β-END1-31
Iβ-END (PERCENT ELUTED)
24
20
16
12
8
4
10
20
30
40
50
60
70
β-END1-26
β-END1-27
β-END1-31
Iβ-END (PERCENT ELUTED)
24
20
16
12
8
4
10
20
30
40
50
60
70
FRACTION

FIGURE 8


A
Ac β-END 1-26
Ac β-END 1-27
B
C
D
Ac β-END 1-26
Ac β-END 1-27
β-END (pmol/fraction)
50
25
20
10
10
5
5
2.5
10
20
30
Fraction